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ORIGINAL ARTICLE

Efficacy of 30% and 38% Silver Diamine Fluoride in Arresting Caries Lesions After Different Application Times: An *in Vitro* Study

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ABSTRACT

Objective: To evaluate the efficacy of silver diamine fluoride (SDF) in arresting dentin caries lesions when applied under different concentrations and times. **Material and Methods:** Forty-two bovine blocks were selected and fixed in 24-well plates. Each well received a mixed bacterial inoculum added to the culture medium with 5% sucrose. The plates were incubated in microaerophilia (7 days) for caries formation, confirmed by micro-CT (M1). SDF was applied over the carious lesions for different times and concentrations (n=6): SDF 30% - immediate removal, 1 minute and 3 minutes; SDF 38%, - immediate removal, 1 minute and 3 minutes. The group without treatment was the control. Then, the samples were again scanned by micro-CT (M2) and submitted to a second cariogenic challenge for 21 days. Then, a final scan was performed (M3). **Results:** Mean pH at the culture medium and lesion depth were compared using Kruskal-Wallis and Wilcoxon tests. 38% SDF showed the lowest metabolic activity of the biofilm. All 38% groups and 30% 1 and 3 minutes did not show an increase in mean lesion depth comparing M3 with M1. However, only 30% 3 minutes and 38% 1 and 3 minutes showed a significant reduction of lesion depth. **Conclusion:** The minimum application time of 30% SDF to arrest dentin caries lesion was 1 minute, while 38% SDF arrested with application and immediate removal.

Keywords: Dental Caries; Cariostatic Agents; Dentin; Tooth Remineralization; X-Ray Microtomography.

Introduction

Dental caries is considered a public health problem, with a large negative impact on the population well-being and quality of life, affecting billions of people worldwide, with four peaks in prevalence during the lifetime: childhood, the beginning of the mixed dentition and also in adulthood, at ages 25 and 70 [1]. The high burden of the disease calls for innovative approaches to increase access to affordable, high-quality dental care.

Lately, silver diamine fluoride (SDF) has gained popularity among non-traditional strategies of dental caries management. SDF is a topical cariostatic agent that effectively prevents and arrests dentin caries lesions in primary and permanent teeth [2,3]. Its application over the tooth surfaces is simple, painless, fast and the intervention is usually well accepted by patients and caretakers [4]. It has also been shown that providing SDF as a caries management strategy for young children has the potential to reduce public expenditures for dental services by avoiding more expensive caries treatment options [5].

The mechanism by which SDF exerts its cariostatic effect is still under debate. However, it is already known that SDF has an antibacterial effect on cariogenic biofilm [6], prevents collagen degradation due to its inhibitory effect on the matrix metalloproteinase [7], and increases the microhardness of dentin [8].

Clinical and laboratory studies have found a dose-response association between fluoride concentration in SDF and its cariostatic effect [7,9]. Not surprisingly, SDF is most commonly used at 38% [10] (i.e., expected fluoride and silver ion concentrations of 44,800 ppm and 200,000 ppm, respectively). However, in Brazil, where this silver compound has been in use for decades, SDF at 30% [10] (i.e., expected fluoride and silver ion concentrations of 35,400 ppm and 255,000 ppm, respectively) is the solution with the highest concentrations of fluoride and silver available on the market. A systematic review showed that SDF at concentrations of 30% and 38% are more effective than other minimal intervention strategies in preventing and arresting caries lesions in dentin [11].

The application time of SDF to tooth surfaces vary across different clinical trials, ranging from 10s [12] to three minutes [13], so there is no consensus in the literature. The protocol of the University of California at San Francisco, USA, recommends allowing SDF to absorb for up to one minute, if reasonable, but mentions that a few seconds of application may be enough to achieve caries arrest in uncooperative patients [14]. Thus, since treatment with SDF is often indicated in non-cooperative children, for patients with phobia of dental treatment and those with cognitive or physical disabilities [14], it is extremely important that the application of this product must be applied quick and effective. However, to date, no study evaluates the minimum time required for application of SDF to be effective, exerting its cariostatic effect in dentin caries lesions. Thus, the present study aims to compare, by means of a micro-CT technique, the efficacy of 38% and 30% SDF in arresting caries lesions *in vitro* after different application times.

Material and Methods

Sample Size

The sample size was calculated using the software Bioestat 5.3 (Instituto Mamirauá, Manaus, AM, Brazil), based on a previous study [15]. The difference in lesion depth before and after treatments were measured by micro-CT in SDF and water treated groups to estimate the parameters. Considering an *in vitro* longitudinal evaluation of the same specimens, based on a two-sided test, pondering a power of 95% and α=1%, a sample size of four dental blocks allocated to each group was required to complete the study. Adding an

estimate of 30% loss of specimens during the assay, it was decided that six blocks would be allocated in each group to complete the study.

Specimen Preparation

Sound bovine incisors (n=40), disinfected in a two percent formalin solution (pH 7.0), were selected after confirming the absence of caries lesions, stains, cracks, or other defects. They were cut at the long axis of the crown using a low-speed saw (Isomet model 11-1280-170, Lake Bluff, IL, USA), resulting in three blocks per tooth, totaling 120 blocks. Each dentin block (4x4x2 mm) was flattened and polished in a metallographic polisher (APL4, Arotec, Cotia, SP, Brazil) as prepared for surface microhardness analyses. Figure 1 illustrates the experimental phases involved in the present study.

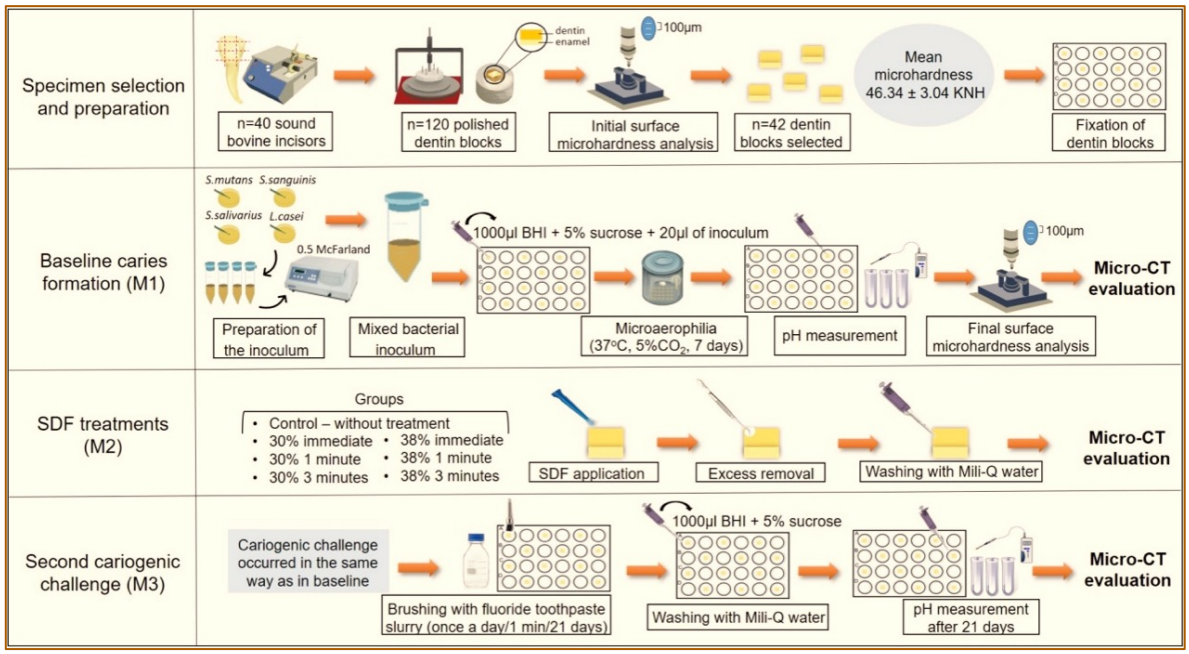

Figure 1. Experimental phases included in the present study.

Baseline Surface Microhardness Analysis for Selection of Dentin Blocks

The polished dentin blocks were analyzed for its initial surface microhardness in a Micromet 5104 (Buehler, Lake Bluff, IL, USA), with a Knoop type diamond penetrator, using a load of 10 g, for five seconds. Three indentations spaced 100 µm apart from each other in the center of each specimen were performed, using a Knoop type diamond penetrator, and the mean microhardness values (KHN) were obtained for each block.

From 120 blocks, 42 with a mean hardness of 46.34 ± 3.04 KNH were selected for the experimental phase and were randomly allocated into the 7 experimental groups (6 treatment groups and a control), with 6 samples per group, as established through the sample size calculation.

After that, they were fixed in polystyrene culture plates (TPP, Zellkultur Testplatte 24F), which were sterilized under UV light for 40 minutes before receiving the microbial inoculum [16].

Preparation of the Microbial Inoculum for Artificial Dental Caries Lesions Formation

Reference strains of *Streptococcus mutans* (ATCC 25175), *Streptococcus Sanguinis* (ATCC 10556), *Streptococcus Salivarius* (ATCC 7073) and *Lactobacillus Casei* (ATCC 393) were re-activated from its original

cultures in Brain Heart Infusion (BHI, Difco, Sparks, EUA) for 48 h at 37 °C, with 5% CO2. After this period, a loopful of bacterial colonies of each specie was collected and suspended in 25 mL of BHI (Difco, Sparks, EUA) to reach an optical density between 0.08 to 0.13, checked in a spectrophotometer (Biospectro SP-220 UV-VIS, Equipar, Curitiba, Brazil) under 625 nm of absorbance. This cell density is equivalent to 1.5×10^8 colony forming units (CFU) per milliliter (CFU/mL), corresponding to a scale of 0.5 of McFarland [17].

Each bacterial sample was stored into four individually identified glass tubes, and after that, one mL of each bacterial sample was put into a single tube, resulting in a mixed bacterial inoculum with a final concentration of $1.5x10^8$ CFU/mL.

Baseline Artificial Dentin Caries Formation (M1)

The induction model for the artificial carious lesions was based on previously published methodologies [16,18,19]. Each culture plate well containing one dentin block received 1000µl of BHI with 5% sucrose and the mixed bacterial inoculum (20µl), giving a final concentration of $3x10^6$ CFU/mL. The plate/blocks system were incubated in microaerophilia for seven days at 37 °C to keep the cells viable to form a mature cariogenic biofilm. The culture media was changed every 24 hours to maintain the viability of bacterial cells [16].

Validity of the Microbial Cariogenic Model

After the cariogenic challenge, the pH of the culture medium in contact with the dentin block was measured to investigate the metabolic activity of the formed biofilm. The culture medium was removed from each well and was stored in individually identified tubes. The pH measurements were performed using an electrode (Orion 9690) connected to a pH meter (Orion Star Series, Thermo Fisher Scientific, Waltham, USA). High pH values mean low acid production and low pH values mean high acid production by bacteria.

Surface Microhardness Analysis

After the baseline caries formation, the surface microhardness measurements were performed, using the same parameters described previously. Three new indentations were made, 150 µm apart from the baseline indentations and spaced at 100 µm from one to another. The percentage of surface hardness loss (% SHL) was used to quantitatively measure the surface demineralization of the specimens, considering the initial and final surface hardness (after M1).

Micro-CT Examination

The blocks were scanned in a high-energy micro-CT (model 1173, Bruker, Kontich, Belgium). They were wrapped in parafilm™ during the scanning procedures to avoid desiccation. Acquisition parameters included: 70 kV, 104 µA, 7.08 µm pixel size, exposure time of 1000 ms, 0.5° rotation step over 360°, frame averaging of 5, and random movements of 20. After this, cross-section images of each specimen were reconstructed using a proprietary software (NRecon, Bruker) and standardized parameters: ring artifact correction of 10, beam hardening correction of 50%, no noise reducing filters, and input of minimum (0) and maximum (0.08) contrast limits. The reconstructed images were saved in grayscale (8-bit) with a BMP format image sequence. The micro-CT scanning was performed in three moments: after M1 – baseline caries formation; M2 - immediately after SDF treatments; M3 - after 21 days of a second cariogenic challenge.

Treatments of Artificial Caries Lesions with SDF

The carious specimens were treated, by a single operator, with SDF solutions at 30% (Cariostop, Biodinâmica, Ibiporã, PR, Brazil) or 38% (Advantage Arrest, Oral Science Brossard, QC, Canada) using three different application times: SDF application and immediate removal; 1 minute application; 3 minutes application. A control group without SDF treatment was also included. In all specimens, SDF was applied with a microbrush (KG Brush Fine, KG Sorensen, Cotia, SP, Brazil) and the excess was removed with a cotton pellet after the application time. Then, the block was washed using 2000 µL of Mili-Q water. All blocks were treated by a single operator. The mean amount of SDF applied to each dentin block was 0.6 ± 0.1mg, estimated by calculating the weight of the microbrush before and after application [6].

High Cariogenic Challenge Simulating Oral Environment (M3)

Soon after SDF treatments, the blocks were submitted to a second microbiological cariogenic challenge following the previously described methodology. In addition, to better simulate the oral environment, the specimens in all groups received a 1450 ppm fluoride toothpaste slurry application (3 mL of water to 1 g of dentifrice, Colgate Maximum Dental Protection Anti Caries Mint) once a day, for 1 minute, during 21 days [20].

The slurry was applied by brushing manually the blocks with a sterile straight Robinson brush, performed daily by a single operator. All procedures were undertaken inside the laminar flow after the removal of the specimen from the culture medium. After, the blocks were washed with 3000 µL of Milli-Q water, and subsequently, a new culture medium with 5% sucrose was added to the plates/blocks system.

The inoculum, as performed previously, was changed every seven days to keep the microorganism viability. In addition, the acidogenicity of the culture medium was again verified at the end of the assay as a surrogate measure of the antibacterial activity of SDF following the same parameters described previously.

Digital Image Analysis

After reconstruction, the three image stacks (after M1, M2 and M3) were co-registered through an affine algorithm applied to the 3DSlicer software [21] to place the three volumes produced for each specimen in the same set of spatial coordinates. Next, the lesions depth was measured at M1, M2 and M3. Caries arrest was considered if M3 lesion depth values were not statistically significantly higher than M1 values. Similarly, reduction of the lesion depth or a possible remineralization was considered if M3 values were statistically significantly lower than M1 values. Finally, a visual analysis of the distribution of high-density areas corresponding to the deposition/distribution of silver was undertaken.

Data Analysis

Data were analyzed in SPSS 21.0 software (IBM, Chicago, IL, USA). The Shapiro-Wilk test was used to verify the normality of the % SHL values, pH measurements and lesion depth at baseline. Kruskall-Wallis, followed by Dunn's test, was used to compare % SHL values, pH distribution and lesion depth among the groups at the different experimental periods. Wilcoxon test was used to compare pH values and lesion depth in each group at different moments. A significance level of 5% was considered for all analyses.

Results

Figure 2 shows the distribution of surface hardness loss percentage among the specimens in the experimental groups after baseline caries formation (M1). All groups showed extensive demineralization, without statistically significant changes among the groups, which validated the artificial caries lesion model used.

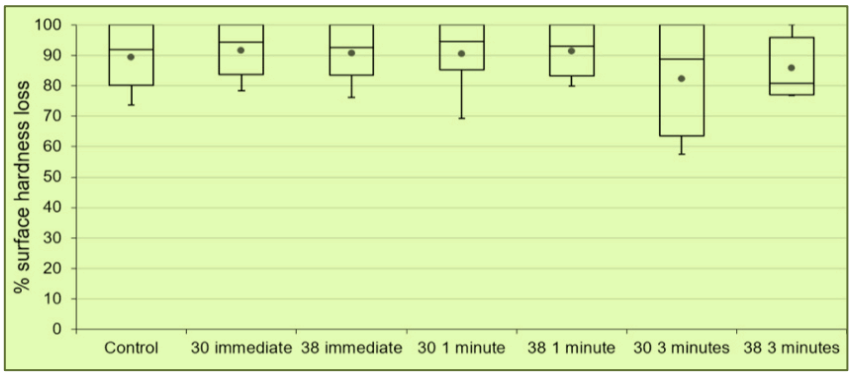

Figure 2. Distribution of surface hardness loss (%SHL) after baseline caries formation (M1). No statistically significant differences were detected among the groups (Kruskall-Wallis).

Table 1 shows the mean pH of the culture medium after baseline caries formation (M1) and after the second cariogenic challenge (M3). The mean baseline pH was 4.88 ± 0.12 for all samples, confirming the high metabolic activity of the biofilm to produce acid. In M3, all groups showed increased pH values than M1 ($p<0.001$). The 38% SDF groups presented the highest mean pH (lower metabolic activity of the biofilm), but without difference compared with 30% SDF and control groups in M3 (Table 1). In addition, the lowest mean pH (higher metabolic activity) was observed in 30% immediate group, but also it was not statistically significant from the other ones in M3.

Table 1. Mean pH of the culture medium among the groups after baseline caries formation and after SDF applications and second cariogenic challenge.

Group	After Baseline Caries Formation (M1)		After SDF and Second Cariogenic Challenge (M3)	
Control	$4.80 \pm 0.10^{a,b}$	$4.80 \pm 0.10^{a,A}$	5.05 ± 0.06^{a}	$5.05 \pm 0.14^{a,B}$
30 immediate	$4.89 \pm 0.05^{a,b}$		4.98 ± 0.07^{a}	
30 - 1 min	$4.93 \pm 0.22^{a,b}$	$4.85 \pm 0.14^{a,b,A}$	5.08 ± 0.06^{a}	$5.06 \pm 0.08^{a,B}$
30 - 3 min	4.74 ± 0.01^{a}		5.12 ± 0.05^{a}	
38 immediate	5.00 ± 0.01^{b}		5.32 ± 0.21^{a}	
38 - 1 min	$4.96 \pm 0.01^{a,b}$	$4.94 \pm 0.06^{b,A}$	5.49 ± 0.10^{a}	$5.33 \pm 0.23^{a,B}$
38 - 3 min	$4.86 \pm 0.01^{a,b}$		5.17 ± 0.28^{a}	
All groups	4.88 ± 0.12^{A}		5.17 ± 0.20^{B}	

Mean values followed by distinct lowercase superscript letters in the same column are statistically different (Kruskal-Wallis followed by Dunn's test, $p<0.05$). Mean values followed by distinct uppercase superscript letters in the same row are statistically different (Wilcoxon test, $p<0.05$).

Table 2 shows that lesion depth was similar among the groups after baseline caries formation (M1) and after SDF application (M2). After the second cariogenic challenge (M3), the control group showed the highest increase in lesion depth, followed by the 30% immediate group. The 30% 3 minutes and 38% 1 and 3 minutes SDF groups showed a lower depth of lesion at M3 compared to M1 ($p<0.05$). Figure 3 illustrates these results. It was possible to see that 38% immediate and 30% 1-minute groups did not show an increase in mean lesion depth at M3 compared to baseline caries formation (M1), indicating caries arrest. However, only 30% 3 minutes and 38% 1 and 3 minutes showed statistically significant lower lesion depth in M3 compared to M1 (Table 2), showing that remineralization of the lesion probably occurred in these groups.

Table 2. Mean lesions depth (µm) after baseline caries formation (M1), immediately after SDF treatment (M2) and after 21 days (M3) of high cariogenic challenge by group.

Group	Lesion Depth at Baseline Caries (M1)	Lesion Depth Immediately After SDF (M2)	Lesion Depth After Second Cariogenic Challenge (M3)
Control	311.62 ± 51.20^{a,A}	254.81 ± 59.04^{a}	576.75 ± 85.53^{a,B}
30 Immediate	292.68 ± 116.62^{a,A}	196.28 ± 102.88^{a}	530.27 ± 168.43^{a,b,B}
30 - 1 min	426.98 ± 203.34^{a,A}	201.44 ± 107.10^{a}	323.67 ± 140.52^{a,b,c,A}
30 - 3 min	315.06 ± 94.39^{a,A}	144.62 ± 142.68^{a}	197.99 ± 142.75^{b,c,B}
38 Immediate	275.46 ± 34.97^{a,A}	191.11 ± 73.55^{a}	218.65 ± 111.72^{a,b,c,A}
38 - 1 min	287.52 ± 73.16^{a,A}	170.45 ± 59.78^{a}	203.15 ± 96.38^{a,b,c,B}
38 - 3 min	273.74 ± 39.60^{a,A}	141.18 ± 47.41^{a}	123.96 ± 35.18^{c,B}

Mean values followed by distinct lower script letters in the same column differ among them at a significance level of 95% (Kruskal-Wallis followed by Dunn's test). Also, mean values followed by different capital letters in the same line represent statistically different results (Wilcoxon test, $p<0.05$).

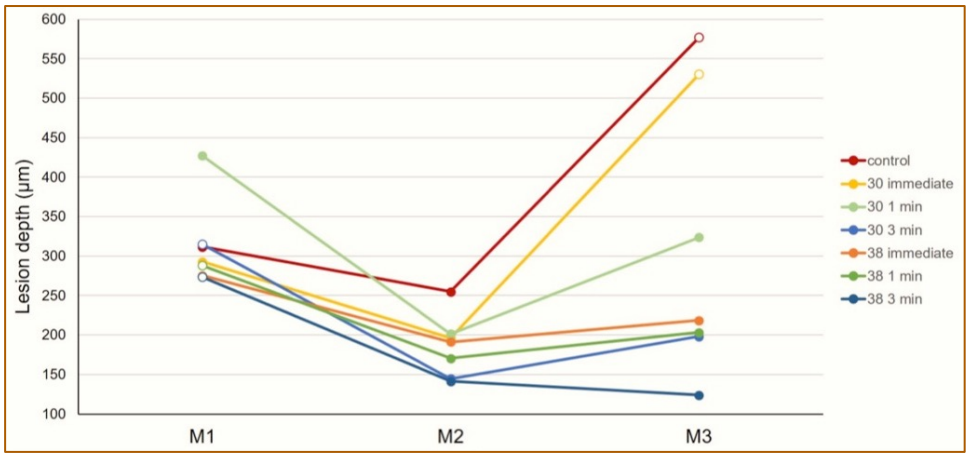

Figure 3. Distribution of lesion depth (µm) after baseline caries formation (M1), immediately after SDF application (M2) and after the second cariogenic challenge (M3) among the experimental groups. Open circles indicate statistically significant differences in lesion depth between M1 and M3 inside the same experimental groups (Wilcoxon test, $p<0.05$).

Representative selected micro-CT slices of all studied groups at M2 and M3 can be visualized in Figure 4.

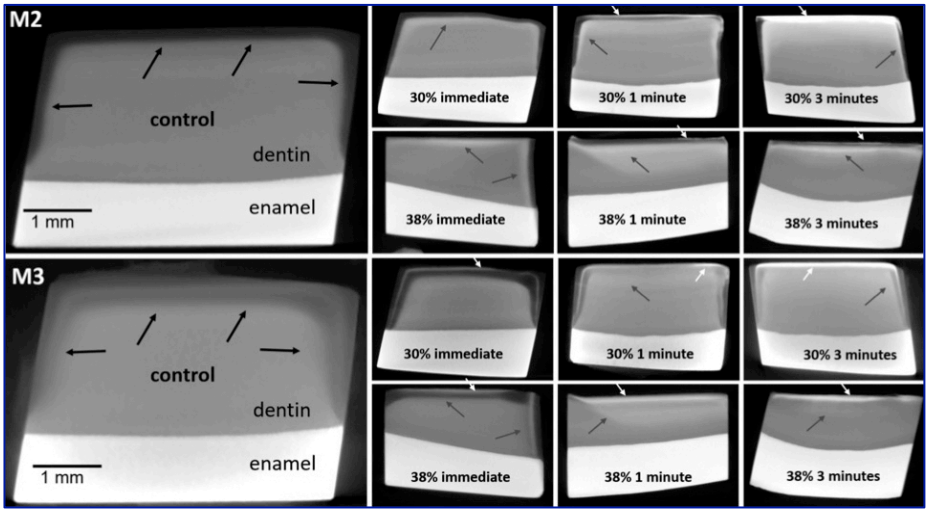

Figure 4. Carious dentin blocks, shortly after treatment with SDF (M2) and after 21 days under high cariogenic challenge (M3) according to the groups of the study. Black arrows in the control group indicate the location of the carious lesion. A highly dense surface layer is indicated by the white arrows, while the grey arrows show a diffuse dense layer.

At M2, the immediate groups showed a diffuse dense layer below the lesion (grey arrows), while both concentrations at 1 and 3 minutes showed a dense surface layer (white arrows). At M3, this surface dense layer is seen in all groups, followed by further lesion progression at the bottom of the immediate groups. We hypothesize that in these groups of immediate application, the dense layer appeared later (at M3), after has been exposed by the daily toothbrushing procedures. For 1- or 3-minutes application groups at M3, the dense surface layer is thicker than at M2 and seemed to have provided more resistance to caries progression.

Discussion

The present *in vitro* study was undertaken to compare different application times and concentrations of SDF to arrest dentin caries lesions. Collectively, our results indicate that both concentrations, with a minimum application time of 1 minute, were able to arrest/slow down further caries progress. However, only the 30% 3 minutes and 38% with 1- and 3-minutes application was able to remineralize the lesion within this *in vitro* caries formation model.

Regarding the present methodology, the authors opted to use dental blocks in a model of caries lesion formation that employed a mature cariogenic biofilm since microbial cariogenic models are more comparable to the *in vivo* caries formation mechanism than chemically induced demineralization models [22]. Furthermore, concerning the micro-CT analysis, all blocks were analyzed following the same coordinates at three different moments to ensure that the depth of the caries lesion was being recorded in the same place in all specimens and experimental periods. Also, the samples were subjected to a high cariogenic challenge and were daily brushed for 21 days, since after this period, it was possible to observe the arrest of caries lesions in dentin treated with SDF [20].

In this *in vitro* study, the authors were also careful to validate the artificial caries formation method by measuring the pH of the culture medium after the experimental period and the surface hardness loss percentage. Although there was a baseline difference among the 38% immediate group and others, all showed a similar percentage of hardness loss and lesion depth, as observed by micro-CT evaluation, which made all groups comparable.

Regarding a possible antibacterial effect of SDF, pH measurements obtained after M3 suggest a lower antibacterial activity after application of SDF, since statistically significant higher pH values in the culture medium were observed in the 30% and 38% SDF groups, all together, at M3 compared to M1. These results are corroborated by others [23], who found statistically significant higher pH at the culture medium after cariogenic challenge for SDF treated tooth surfaces compared to control and NaF treated groups, suggesting that F and Ag present in SDF have a strong effect on the inhibition of acid production by *Streptococcus mutans*, thus inhibiting their metabolic activity. Furthermore, although no *in vitro* study to date has simulated the oral environment with toothbrushing for 21 days, a recent study evaluated the mechanical effect of brushing on demineralized surfaces treated with 38% SDF and found that after 7 days of brushing, the SDF-treated dentin remained with antibacterial properties [24].

It was possible to note that application of the SDF 30% and its immediate removal was not able to arrest further caries development. Thus, at this concentration, the application time should be for 1 or 3 minutes. In fact, the progression of caries was arrested in all groups except this. However, a possible lesion remineralization was only observed in the 30% 3 minutes and 38% 1- and 3-minutes application groups. In these specimens, a statistically significant reduction in lesion depth was seen when M1 and M3 were compared. In contrast, a recent *in vitro* study that also used micro-CT to investigate whether SDF at concentrations of

30% and 38% applied for 1 and 3 minutes would be able to remineralize blocks of dental enamel from deciduous teeth the authors observed that SDF 30% promoted greater enamel remineralization compared to other times and concentrations [25]. However, in the mentioned study, the samples were not subjected to a cariogenic challenge after treatment with SDF, and the way of remineralization evaluation was the quantification of the internal porosity of enamel (the fewer pores, the greater the remineralization). In the present study, this assessment was carried out by measuring the depth of the caries lesion in dentin by micro-CT.

Although it is not completely clear how SDF contributes to the increase of the hardness of the caries lesion, some authors proposed that it happens due to a reaction between silver and dentin minerals, rather than classic fluoride mediated remineralization [8]. The results of the present study support this hypothesis since the micro-CT technique revealed a highly dense layer beneath the carious lesion. Furthermore, Srisomboon et al. [26] also applied 38% SDF at different times (30s, 60s and 180s) to investigate its effect on mineral precipitation of demineralized dentin and found, through the scanning electron microscope, that at all times of application, the peritubular dentin and the dentinal tubules of the specimens were filled with precipitated crystals such as calcium phosphate and crystals containing Ag and Cl. However, an important limitation mentioned by these authors is that it was not possible to mimic the oral environment, and, in addition, they suggest that new studies with an application time of less than 30s should be performed [26]. Thus, future studies that carry out further elemental analysis of caries lesions are necessary to investigate and confirm the remineralizing effect of SDF.

Our results corroborate the findings of a previous micro-CT study in which, after application of 38% SDF, a distinct opaque and dense layer on the surface of the lesion was visualized [27]. Others highlighted the presence of a highly dense layer surrounding the bottom of the dentin cavitated lesion immediately after SDF application [28]. However, it is also important to emphasize that only the groups that received SDF for 1 and 3 minutes, in both concentrations, presented a high dense surface layer. After 21 days under a high cariogenic challenge, this layer further occupied some of the lesion extent. Previous authors showed that after treatment of demineralized dentin with 38% SDF, a penetration of Ag was observed over the entire demineralized surface and in the healthy underlying dentin, which was better observed after 1 year of treatment of these samples. Thus, it means that as time went by, the maturation and detection of Ag crystals increased [29]. Another possible explanation for the increase in this high dense surface layer could be the further mineral rearrangement in the lesion during the second cariogenic challenge and the daily fluoride contacts, but further investigations are warranted.

Micro-CT is an ideal technique for volume and mineral density evaluation of hard tissues [30], considering the non-destructive nature and the possibility of a long follow-up period using the same specimens [16]. The results of this study should, however, be interpreted with caution since some high-density compounds (such as silver) may result in artefact formation. For example, depending on the amount and concentration of silver particles, high-density regions in the specimens can easily result in scattered streaks and pseudo-high-density regions that could be erroneously considered silver deposition.

The 38% formulation of SDF has been successfully used to arrest caries with an application time of up to two minutes in primary teeth of children awaiting definitive treatment at a community clinic [4]. Although the present *in vitro* study has shown similar results for caries arrest at application times of a minimum of 1 minute for 30% and immediate application for 38%, even when exposed to further cariogenic environment, new clinical studies should be performed to observe arrest of dentin caries lesions under different application times.

Conclusion

The minimum application time of 30% SDF to arrest dentin caries lesion was 1 minute, while 38% SDF was able to arrest it with the application and immediate removal. Furthermore, only 30% SDF for 3 minutes and 38% SDF for 1 and 3 minutes resulted in a significative reduction of the lesion depth. This is certainly relevant in terms of identification of thresholds for clinical effectiveness of SDF therapy. Further clinical studies, with long-term follow-up, should be performed to confirm the present results in clinical settings.

Authors' Contributions

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All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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